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On the systematics and the phylogenetic position of the poorly known, montane dragon-lizard species *Pseudocalotes austeniana* (Annandale, 1908) (Squamata, Agamidae, Draconinae)

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Abstract

The montane agamid species *Pseudocalotes austeniana* has had a complicated taxonomic history, as the species was initially described as a member of the genus *Salea* Gray, 1845. Later, the species was placed in a monotypic genus *Mictopholis* Smith, 1935, which was erected only to include this species; however, the species was later on transferred to the genus *Pseudocalotes* Fitzinger, 1843, owing to the morphological similarities, and lack of strong characters to diagnose the genus *Mictopholis*. Nonetheless, its precise phylogenetic and systematic position has remained unresolved due to the lack of molecular sequence data. During a herpetological expedition to Arunachal Pradesh, specimens of *P. austeniana* were collected from the hills near the type locality. The mitochondrial 16S rRNA, ND2 and ND4, and the nuclear RAG1 regions were subjected to molecular phylogenetics. Maximum Likelihood and Bayesian Inference gene trees revealed that *P. austeniana* is a member of the subfamily Draconinae. The analyses showed that the genus *Pseudocalotes* is polyphyletic, and *P. austeniana* was embedded within the genus *Japalura* Gray, 1853 sensu stricto. We here, thus, propose to transfer the species *P. austeniana* to the genus *Japlaura*, as *Japalura austeniana* **comb. nov.** Biogeographic and evolutionary significance of the findings are discussed.

Key Words

Arunachal Pradesh, generic re-allocation, *Japalura*, Himalayas, northeast India

Introduction

The montane agamid species *Pseudocalotes austeniana* (Annandale, 1908) has had a complicated taxonomic history. Annandale (1905) examined two specimens of agamid lizards from the northeast India, deposited at ZSI, one, collected by Dr. Stoliczka from Lower Burma (now Myanmar), and the other (ZSI R 3976), collected by Colonel Godwin-Austen from Harmatti (now Harmuti), near Itanagar, while on an expedition in the Dafla Hills of Assam, which he assigned to *Salea horsefieldii* (not of Gray, *fide* Smith 1935). In a subsequent communication, Annandale (1908) retained the specimen from Lower Burma under *Salea horsefieldii*, however, he described the specimen collected by Godwin-Austen from Dafla Hills as a new species, *Salea austeniana*. The new species was diagnosed as distinct from *S. horsefieldii* in the presence of a fold in front of the shoulder and in the proportions of the

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head region (Annandale 1908). Smith (1935) in his revision of the lizard fauna of British India, erected a new genus, *Mictopholis* Smith, 1935 to accommodate this species. He provided a short diagnosis, in that he neither explicitly justified the necessity for such taxonomic arrangement, nor sufficiently defined the genus (Mahony 2010). With the difficulty posed by the terrain for herpetofaunal explorations, the species was never seen or reported again, as indicated by the lack of specimens deposited after Annandale's description (1908), till 2007 (Das and Das 2007), and only appeared in Moody's (1980) unpublished thesis, and in numerous checklists (*fide* Das and Das 2007). The species was rediscovered by Das and Das (2007) from Sangti, West Kameng District, Arunachal Pradesh, who added two specimens, currently housed at ZSI (male, ZSI 24841, Bomdila) and MAVC (female, MAVC L31, Dirang), increasing the number of specimens from one to three, and provided details on the female colouration of the species for the first time. The species was recently reported from China (Wang et al. 2019), expanding the distribution range by 400 km northeastward, adding three more specimens to the collection. It is known to occur with certainty in the Dafla hills, from Harmatti, Bomdila and Dirang (Das and Das 2007), Eaglenest Wildlife Sanctuary (Athreya 2006; Agarwal et al. 2010) and Medog County, southeastern Tibet (Wang et al. 2019) only, and the geographic distribution *P. austeniana* remains poorly understood.

Mahony (2010), attributing to lack of strong morphological characters to define the genus *Mictopholis*, synonymized *Mictopholis* to *Pseudocalotes*, and stated that phylogenetic analysis of the subfamily Draconinae was necessary to comment on the systematics of *P. austeniana*.

During a herpetological expedition to Arunachal Pradesh, we collected specimens of *P. austeniana* (Fig. 1) from Manipolyang (27.5247°N, 93.8620°E, ca. 1700 m.), Lower Subansiri district, Arunachal Pradesh, about 30 km aerially from the type locality. The freshly collected specimens allowed us to get molecular voucher and to analyze phylogenetic relationship of this charismatic montane lizard with the other taxa within the subfamily Draconinae. Based on the tree topologies obtained from Maximum Likelihood and Bayesian Inference phylogenetic reconstructions, which reveal the phylogenetic position of the species within the subfamily Draconinae, we here elucidate the generic position of *P. austeniana*.

Materials and methods

Institutional abbreviations used in this study

BNHS – Bombay Natural History Society, Mumbai; CES – Centre for Ecological Sciences, Bengaluru; MAVC – Museum of Arya Vidyapeeth College, Guwahatai, Assam; ZSI – Zoological Survey of India, Kolkata, West Bengal.

Figure 1. A male *Japalura austeniana* comb. nov. specimen (CESL1212), collected from Manipolyang, Lower Subansiri district, Arunachal Pradesh, in life.

Sample collection and preservation

Two specimens, an adult male (CESL1212) and a juvenile (CESL1204), were collected by hand and euthanized using halothane, after which, they were fixed in 6% formaldehyde solution for 48 hours. The fixed specimens were washed with water to remove traces of formaldehyde, following which, they were transferred to 70% ethanol for long-term preservation. Thigh muscle tissue was taken from the specimens immediately after euthanasia, and the tissues were stored in ~99.9% molecular grade ethanol for DNA extraction. The specimens and the tissues were deposited at the collections facility at CES. The specimens were photographed in life prior to euthanasia, and in preservation using Canon 100 mm macro lens, mounted on Canon 70D, illuminated with two Canon 430EX-II external flashes (Canon Inc., Tokyo, Japan).

DNA isolation, amplification and sequencing

Total genomic DNA was isolated from the preserved muscle tissues by using Qiagen DNAeasy kits following protocols provided by the manufacturer. A fragment each of the three mitochondrial 16S rRNA gene (16S), Nicotinamide Adenine Dinucleotide Hydrogenase (NADH) subunit 2 (ND2), NADH subunit 4 (ND4) gene and one nuclear Recombination Activating Gene 1 (RAG1) was amplified using published primers (Harvey et al. 2017; Pal et al. 2018; Ambekar et al. 2020). A 22-µl reaction was set containing 10 µl of Thermo Scientific DreamTaq PCR Master Mix, 9 µl water, 0.5 µl of each primer, and 2 µl template DNA, carried out with an Eppendorf Mastercycler Nexus GSX1. Thermo-cycles used for amplification were as follows: 94°C for 5 min (denaturation temperature 94 °C for 30 s, annealing temperature 45 °C for 30 s for 16S, 60 °C for 50 s for ND2, 63 °C for 30 s for ND4, 58 °C for 50 s for RAG1, elongation temperature 72 °C for 1 min) × 30 cycles, 72 °C for 10 min, hold at 4 °C. PCR product was cleaned using a QIAquick PCR Purification Kit and sequenced with an AB 3730 DNA Analyzer. The sequences were visualized in Chromas, edited, and aligned in MEGA v. 6.

We downloaded the relevant mitochondrial genomic sequences by Shaney et al. (2020), particularly for ND4 dataset, which included those of the members of the genus *Pseudocalotes* sensu lato. Sequences of the 16S, ND2, ND4 regions from the genomic sequences were extracted manually. Additionally, published 16S, ND2, ND4 and RAG1 sequences of multiple species representing the genera within Draconinae and Agaminae available on GenBank (Benson et al. 2017) were downloaded for use in molecular phylogenetic purposes (Suppl. material 1). Taxon selection largely followed Wang et al. (2018). The datasets for each genetic fragment were aligned separately in MEGA v. 6 (Tamura et al. 2013) using CLUSTALW (Higgins et al. 1994) and separate gene trees were inferred for each dataset to test for congruence between the gene trees and to test the possibility of concatenation. Given that there were generally no incongruences between the gene trees, the mitochondrial 16S, ND2 and ND4 datasets were concatenated. However, given the limited proportion of species for which RAG1 sequences were available and the lack of correspondence between the specimens from which each of the genes were sequenced, the nuclear RAG1 dataset was analyzed separately. The final concatenated, 2062 base pair (bp) long mitochondrial dataset included 60 sequences representing 53 species belonging to 26 genera.

Molecular phylogenetics

Maximum Likelihood (ML) and Bayesian Inference (BI) trees based on the concatenated mitochondrial dataset were inferred to assess the relationships within the genus *Pseudocalotes* and the subfamily Draconinae, in general, and to determine the phylogenetic position of *P. austeniana* within the subfamily. The coding ND2 and ND4 regions were partitioned per-codon position, whereas the non-coding 16S region was not partitioned per-codon position, ML trees were reconstructed using W-IQ-TREE (Trifinopoulos et al. 2016), the web implementation of IQ-TREE (Nguyen et al. 2015). Branch support was tested using ultrafast bootstrap (UFboot) analysis (Minh et al. 2013), performing 1000 ultrafast bootstrap alignments. Model of sequence evolution to be implemented was tested using MODELFINDER (Kalyaanamoorthy et al. 2017). For BI analysis, the optimum partitioning strategy and the model of sequence evolution to be employed were determined using PARTITIONFINDER v.1.1.1 (Lanfear et al. 2012). Bayesian Inference trees were reconstructed in MRBAYES v.3.2.6 (Ronquist et al. 2012), two independent analyses were run starting from different, random starting trees. The analyses were run for 50 million generations, and Markov Chains were sampled every 1000 generations. Three heated and one cold chain was used in the analyses. The average standard deviation of split frequencies by the end of the analyses was below 0.005, and the analyses were terminated. The best fitting tree representing the evolutionary relationship was selected based on the 50% majority consensus rule. *Phrynocephalus* spp. were used as the outgroup to root the ML and BI trees. Preliminary 16S (456 bp), ND4 (577 bp), ND2 (1019 bp) and RAG1 (1020 base pair) ML and BI gene trees using the representative species of the Draconinae genera were inferred using more or less the same methodology, as for the concatenated dataset, to test the congruence prior to concatenation.

The partitioning schemes and the model of sequence evolution used for each partition, for all genes and their partitions, across all analyses, is summarized in Table 1.

Results

The ML and BI phylogenetic trees inferred from the mitochondrial ND4 region (Figure 2) revealed the polyphyletic

Table 1. Optimum partitioning scheme and model choice for each partition. *BI analyses had only 6 partitions instead of 7 for the mitochondrial dataset, as the codon position ND4 2nd was merged with 16S at partition 4; whereas in the nuclear dataset the codon position RAG1 3rd was merged with RAG1 2nd.

Partition	Codon position	Model	
Mitochondrial		**ML**	**BI**
1	ND2 1st	GTR+I+G	GTR+I+G
2	ND2 2nd	TN	GTR+I+G
3	ND2 3rd	TN+I+G	GTR+I+G
4	16S	TIM2+I+G	GTR+I+G
5	ND4 1st	TIM3+I+G	GTR+G
6	ND4 2nd	TVM+G	*
7	ND4 3rd	K3Pu+I+G	HKY+I+G
Nuclear		**ML**	**BI**
1	RAG1 1st	TIM3+G	HKY+G
2	RAG1 2nd	TIM3+G	K80+G
3	RAG1 3rd	TIM3+G	*

nature of the genus *Pseudocalotes*. Members of the genus appeared to cluster into two well-supported distinct, major clades, one composed of the species *P. baliomus* Harvey, Shaney, Hamidy, Kurniawan & Smith, 2017, *P. cybelidermus* Harvey, Hamidy, Kurniawan, Shaney & Smith, 2014, *P. guttalineatus* Harvey, Hamidy, Kurniawan, Shaney & Smith, 2014, *P. rhammanotus* Harvey, Hamidy, Kurniawan, Shaney & Smith, 2014, and the type species *P. tympanistriga* (Gray, 1831) (ML ultrafast bootstrap values 100, BI posterior probability 1.00), the other clade was composed of *P. brevipes* (Werner, 1904), *P. kakhienensis* (Anderson, 1879), *P. kingdonwardi* (Smith, 1935), *P. larutensis* Hallermann & McGuire, 2001, *P. microlepis* (Boulenger, 1888) (ML ultrafast bootstrap values 99, BI posterior probability 1).

Pseudocalotes austeniana, nevertheless, did not cluster with any of the two clades, and was instead recovered as sister to *Draco* spp. (ML ultrafast bootstrap values 91, BI posterior probability 0.99). The ML and BI phylogenies inferred from the concatenated dataset were used to elucidate the position of *P. austeniana* within the subfamily Draconinae. The species was recovered as a member of the genus *Japalura* sensu stricto, sister to a clade containing the species *J. kumaonensis* Annandale, 1907, *J. tricarinata* Blyth, 1853 and the type species *J. variegata* Gray, 1853. The relationship was well-supported in the ML analysis (ultrafast bootstrap value 88), but less strongly in the BI analysis (posterior probability 0.81). The (*Ptyctolaemus* + (*Japalura* + *Draco*)) relationship was very well supported (ML ultrafast bootstrap values 100, BI posterior probabilities 1). In the most recent revision of *Japalura* sensu lato, Wang et al. (2018) phylogenetically define *Japalura* sensu stricto as the genus including the species that share a more recent common ancestor with *Japalura variegata* than with *Draco volans* Linnaeus, 1758 or *Ptyctolaemus gularis* Peters, 1864. Based on the molecular phylogenetic reconstructions, and following the phylogenetic definition of *Japalura* sensu stricto by Wang et al. (2018), the placement of *P. austeniana* within the genus *Pseudocalotes* is untenable, and we propose to transfer the species to the genus *Japalura*, as *Japalura austeniana* comb. nov. By implication, we transfer the genus *Mictopholis* from the synonymy of *Pseudocalotes* to *Japalura*. *Japalura andersoniana* Annandale, 1905 was sister to a clade containing all the species of *Japalura* sensu stricto, including *J. austeniana* comb. nov. (ultrafast bootstrap values 58, posterior probability 0.90). The relationships between the ingroup taxa were generally consistent with the previously published phylogenies including Pyron et al. (2013), Grismer et al. (2016), Harvey et al. (2017), Giri et al. (2019), Wang et al. (2019), however, there were certain deviations, particularly with the placement of the genera *Acanthosaura* Gray, 1831, *Cristidorsa* Wang, Deepak, Datta-Roy, Lin, Jiang, Che & Siler, 2018, and *Salea*. Additionally, previous studies have recovered *Ptyctolaemus* spp. as sister to *Japalura* spp., however, in our analyses, we obtained members of the genus *Draco* as sister to those of *Japalura* sensu stricto, with excellent supports (ML ultrafast bootstrap 100, BI posterior probability 1). Further, there were minor differences in the ML and BI tree topologies (Figs 3, 4), nonetheless, both the phylogenetic reconstruction methods consistently placed *P. austeniana* with the members of the genus *Japalura* sensu stricto, and never within *Pseudocalotes* sensu stricto The nuclear RAG1 gene trees topology was in general agreement with those inferred using the combined dataset in the placement of *Japalura austeniana* comb. nov. with *J. tricarinata* (Suppl. material 2).

Discussion

The genus *Pseudocalotes* was found to be polyphyletic (Figs 2–4) as seen in an earlier study (Grismer et al. 2016; Harvey et al. 2017; Shaney et al. 2020). Members of the genus formed two distinct clusters, one composed of *P. baliomus*, *P. cybelidermus*, *P. guttalineatus*, *P. rhammanotus*, and the type species *P. tympanistriga*, which we refer to as the *Pseudocalotes* sensu stricto clade. This clade was sister to the clade containing the genera *Dendragama* Doria, 1888, *Lophocalotes* Günther, 1872 and *Phoxophrys* Hubrecht, 1881. On the other hand, the remaining members of the genus, *P. bapoensis*, *P. brevipes*, *P. drogon*, *P. flavigula*, *P. kakhienensis*, *P. kingdonwardi*, *P. larutensis* and *P. microlepis*, formed a separate cluster, which was sister to the clade containing the genus *Diploderma* Hallowell, 1861, in consistency with Wang et al. (2018) (Figs 2–4). *Pseudocalotes austeniana*, however, did not belong to any of these clades, and was instead found to cluster with *Japalura* spp.. Harvey et al. (2017) noted that although they observed polyphyly in the genus *Pseudocalotes* sensu lato, the study was based only on nine out of 22 known species of *Pseudocalotes*. Even with the addition of molecular data by Shaney et al. (2020), and the rise in the number of species included in the phylogenetic analyses to 14, excluding *Japalura austeniana* comb. nov., the genus continues to remain polyphyletic, if not, paraphyletic, with the exclusion of *Japalura austeniana* comb. nov.

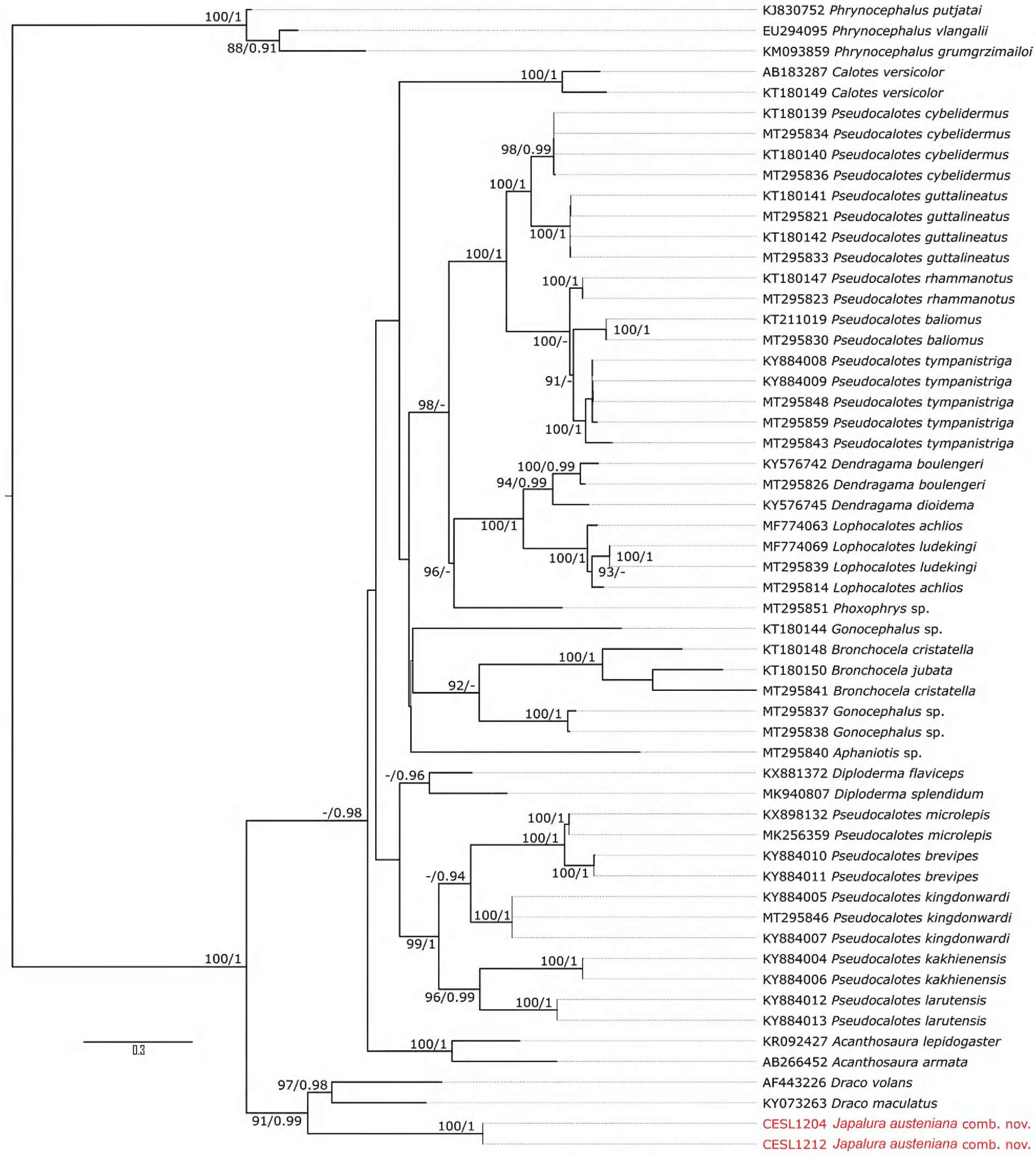

Figure 2. ML+BI phylogenetic tree based on the 577 bp long mitochondrial ND4 dataset, built under the models TIM3+F+G4, GTR+F+I+G4, TPM3u+F+I+G4 (ML) and GTR+G, GTR+I+G and HKY+I+G (BI) for codon positions 1, 2 and 3 respectively. Numbers at the nodes indicate ML ultrafast ultrafast bootstrap values/BI posterior probabilities.

It seems to be necessary to objectively define the genus *Pseudocalotes* sensu stricto. It appears that the genus *Pseudocalotes* is used by authors as per convenience, and the species which cannot be objectively allotted to specific genera are tentatively placed in this genus. Most species of the *P. kakhienensis* clade were earlier placed in a genus different than *Pseudocalotes*, but were later transferred to *Pseudocalotes*. For example, *P. kaulbacki* (Smith, 1937), a junior subjective synonym of *P. kingdonwardi* (Smith, 1935) was described as a member of *Japalura*. *Pseudocalotes kakhienensis* (Anderson, 1879) has been variously placed in the genera *Oriocalotes*, *Acanthosaura*, *Calotes*, and *Salea*, until it was transferred to *Pseudocalotes* (Mahony 2010). Another potential hindrance to the objective definition of the genus *Pseudocalotes* sensu stricto is that until recently, only ND4 sequences of the type species of *Pseudocalotes* were available. However, with the avail-

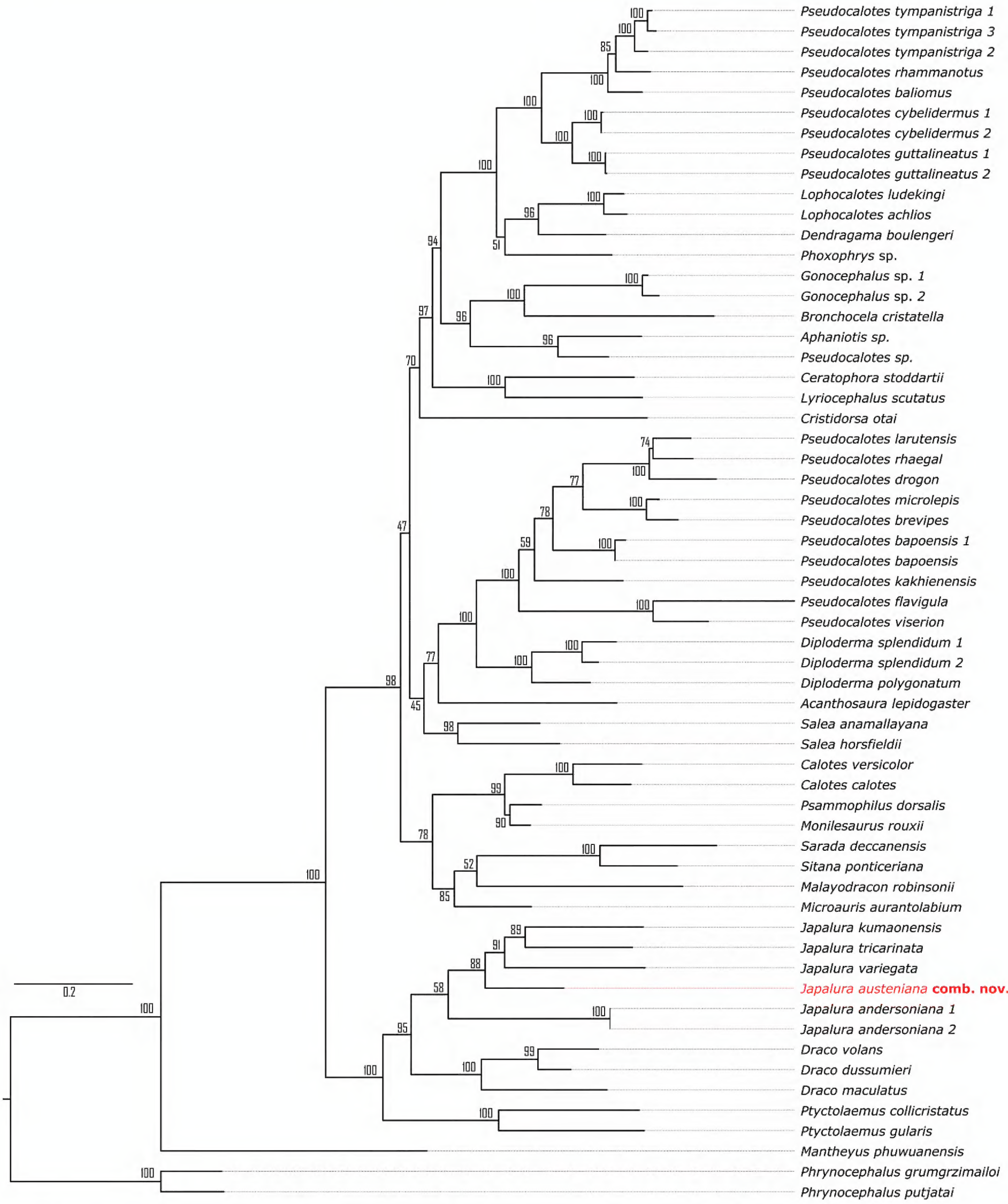

Figure 3. Maximum Likelihood phylogenetic tree based on the 2062 bp long mitochondrial (ND2+16S+ND4) concatenated dataset revealing the phylogenetic position of *Japalura austeniana* comb. nov. Numbers at the nodes indicate ML ultrafast bootstrap values.

ability of mitochondrial genomic sequences by Shaney et al. (2020), we strongly advocate for a systematic revision of the genus *Pseudocalotes* sensu lato with the integration of molecular and morphological data. Further, it is worth mentioning that the assignment of *J. bapoensis* to the genus *Pseudocalotes* needs revaluation. Based on our phylogenetic relationships, the species belongs to *P. kakhienensis* clade, along with *P. brevipes*, *P. drogon*, *P. flavigula*, *P. kakhienensis*, *P. larutensis*, *P. microlepis* and *P. viserion* and the generic allocation of the members of the *P. kakhienensis* clade needs verification. According to the phylogenetic definition of *Pseudocalotes* by Wang et al. (2018), it is a genus which includes species that share a more recent common an-

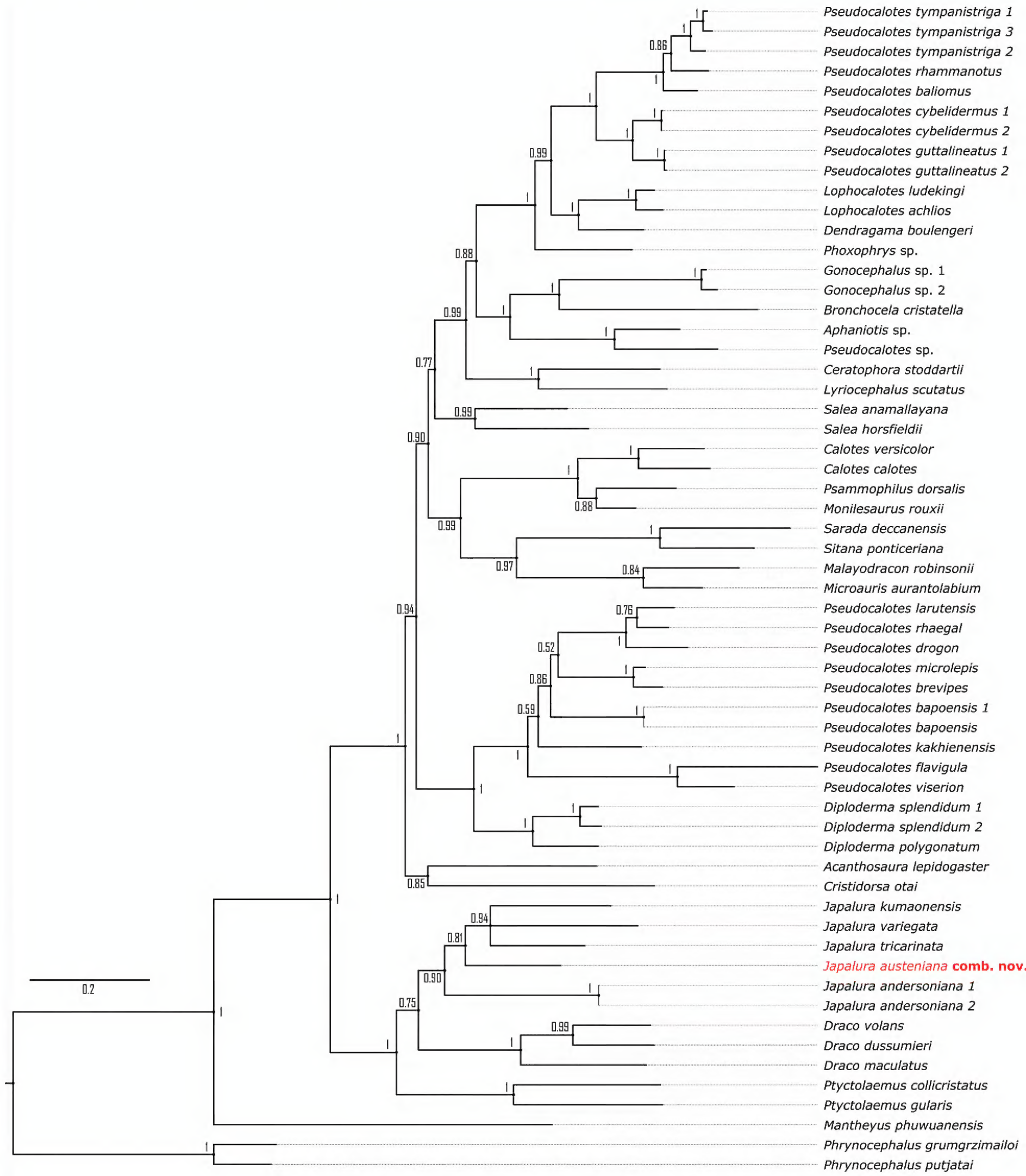

Figure 4. Bayesian Inference phylogenetic tree based on the 2062 bp long mitochondrial ND2+16S+ND4 concatenated dataset revealing the phylogenetic position of *Japalura austeniana* comb. nov. Numbers at the nodes indicate BI posterior probabilities.

cestor with *Pseudocalotes tympanistriga* than with *Diploderma polygonatum* Hallowell, 1861 and *Acanthosaura lepidogaster* (Cuvier, 1829), however, based on the phylogenetic relationships obtained here, it appears that the *kakhienensis* clade is sister to *Diploderma* spp., and it is placed distantly from the *Pseudocalotes* sensu stricto members (Figs 3, 4). From the recently published molecular phylogenies (Harvey et al. 2017, Shaney et al. 2020), it is observed that the genus *Pseudocalotes* is predominantly Javan and Sumatran, with the Indo-Chinese species representing altogether a genus different from *Pseudocalotes* sensu stricto. With the exclusion of *Japalura austeniana* comb. nov. from *Pseudocalotes*, the genus *Pseudocalotes* sensu stricto now is absent from India mainland. A single species, *Pseudocalotes andamanensis* (Boulenger, 1891) occurs on the Andaman and Nicobar Islands of India, however, its phylogenetic position needs to be tested using molecular data.

Mahony (2010) placed *Japalura austeniana* comb. nov. in the genus *Pseudocalotes*, owing to the morphological similarities with the members of the genus, based on an expanded definition of *Pseudocalotes* (*sensu* Hallermann and Böhme 2000), and morphologically compared the species with members of *Pseudocalotes* sensu lato. He, nonetheless, also mentioned that the species was similar to members of the genus *Japalura*, especially with regards to the heterogeneous-sized ventral scales. Wang et al. (2018), in their key to the genera of the subfamily Draconinae, retained the genus *Mictopholis* and placed it closer to *Japalura* sensu stricto than to *Pseudocalotes*, however, did not comment upon the taxonomic status of the genus *Mictopholis*, likely due to the absence of molecular data.

With the increase in the availability of molecular sequence data for multiple species of the agamid subfamily Draconinae, our understanding of the systematic positions and the evolutionary relationships between the genera and their species is rapidly improving, as has been recently evidenced by the description of multiple new genera from south and southeast Asia (Denzer et al. 2015; Deepak et al. 2016; Pal et al. 2018; Wang et al. 2018), and the synonymization of certain other genera (Deepak et al. 2015; Giri et al. 2019). By generating sequence data for yet another montane Draconinae species *Japalura austeniana* comb. nov., and elucidating its phylogenetic position, we further add to the understanding of the phylogenetic relationships within Draconinae. We believe that further herpetological expeditions in the underexplored northeast India are necessary, since the area is poorly explored, and many more species found in northeast India remain to be described. Furthermore, molecular data that are missing for some *Japalura* spp. will be necessary to ascertain the validity of the genus *Mictopholis*. Similarly, we also hope that our understanding of Draconinae systematics will certainly improve with the inclusion of sequences of the species for which molecular data are currently unavailable.

Furthermore, northeast India lies at the interface of southeast Asia and mainland India, thus, enriching the molecular and the morphological databases, especially using the taxa from northeast India and adjoining Himalayan regions is of extreme importance, as these may be useful in testing hypotheses pertaining to the origin and dispersal, into or Out-of India, of the lizards of the subfamily Draconinae.

Conclusions

Molecular phylogenetic reconstructions reveal that the genus *Pseudocalotes* is polyphyletic in nature. The montane agamid species *Pseudocalotes austeniana* was not recovered as a member of the genus *Pseudocalotes*, but instead was found to cluster with *Japalura* spp. The position of *P. austeniana* within *Pseudocalotes* is untenable, and we propose to transfer the species to *Japalura* sensu stricto as *Japalura austeniana* comb. nov.

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Supplementary material 1

A list of species, their corresponding sequences and accession numbers used in this study

Authors: Gaurang G. Gowande, Harshal S. Bhosale, Pushkar U. Phansalkar, Mandar Sawant, Zeeshan A. Mirza

Data type: phylogenetic, genomic, specimen

Explanation note: A table containing a list of species, the corresponding specimens and GenBank(R) accession numbers for each of the genes used in the phylogenetic analyses.

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Supplementary material 2

Figure S1

Authors: Gaurang G. Gowande, Harshal S. Bhosale, Pushkar U. Phansalkar, Mandar Sawant, Zeeshan A. Mirza

Data type: phylogenetic, images phylogenetic, images

Explanation note: ML+BI tree topology based on 1020 bp long nuclear RAG1 dataset.

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